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NOTES

Immunogenic Peptide Comprising a Mouse Hepatitis Virus A59 B-Cell Epitope and an Influenza Virus T-Cell Epitope Protects against Lethal Infection

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The coronavirus spike protein S is responsible for important biological activities including virus neutralization by antibody, cell attachment, and cell fusion. Recently, we have elucidated the amino acid sequence of an S determinant common in murine coronaviruses (W. Luytjes, D. Geerts, W. Posthumus, R. Meloen, and W. Spaan, J. Virol. 63:1408–1412, 1989). A monoclonal antibody directed to this determinant (MAb 5B19.2) protected mice against acute fatal infection. In this study, BALB/c mice were immunized with a synthetic peptide of 13 amino acids corresponding to the binding site of MAb 5B19.2, which was either extended with an amino acid sequence of influenza virus hemagglutinin or conjugated to keyhole limpet hemocyanin. Both immunogens induced S-specific antibodies in mice, but only the hemagglutinin-peptide construct protected them against lethal challenge. In contrast to mouse hepatitis virus type 4 (MHV-4), MHV-A59 was not neutralized in vitro by MAb 5B19.2. Neither MHV-A59 nor MHV-4 was neutralized in vitro by antibodies comprising by the synthetic peptides. Our results demonstrated that antibodies elicited with a synthetic peptide comprising a B-cell epitope and a T-helper cell determinant can protect mice against an acute fatal mouse hepatitis virus infection.

The pathogenesis of the murine coronavirus mouse hepatitis virus (MHV) has been extensively studied over the past decade (20, 24). In addition to hepatitis, MHV causes encephalitis in the natural host (20, 25); by its ability to provoke subacute demyelination, the infection is a useful animal model for similar conditions in humans (W. J. M. Spaan, D. Cavanagh, and M. C. Horzinek, *in* M. H. V. Van Regenmortel and A. R. Neurath, ed., *Immunochemistry of Viruses II*, in press). The coronavirus spike protein (S) is an important determinant of coronavirus virulence and tissue tropism (2, 3); it is responsible for important biological activities including virus neutralization by antibody, induction of a cellular immune response, attachment to the target cell, and cell fusion (23; Spaan et al., in press). Variants of MHV which escape neutralization and recombinants containing hybrid S genes (strains MHV-4 and -A59) can be of reduced neurovirulence (4, 5, 12), which has been seen also after passive immunization of mice with neutralizing S-specific monoclonal antibodies (MAbs) (2). In all these cases, the normally acute encephalitis was suppressed, and in some of these cases, a chronic demyelinating disease was induced instead. Mice immunized with a synthetic decapeptide homologous to a domain in the C-terminal one-fifth of the S protein of MHV-4 (JHM) were partially (76%) protected from challenge with 10 PFU of virus, and only 44% were protected against 100 PFU. Thirty-five percent of mice immunized with keyhole limpet hemocyanin (KLH) carrier alone survived a 10-PFU challenge (22). The amino acid sequence of the neutralizing MAb 5B19.2 binding site A has recently been elucidated (16). It is conserved in most murine coronaviruses (21), which suggests that it is part of an important functional domain.

In the present report, we demonstrate that synthetic peptides representing binding site A induce an S-specific antibody response; more important, they will protect mice against a lethal intracerebral challenge infection.

Immunogenicity of synthetic peptides. Peptide E2 representing binding site A possesses the amino acid sequence H_2N-846-SPLLGCIGSTCAE-858-COOH of the S protein of MHV-A59 (17); upon synthesis (18), one-half of the resin including the peptide was further processed for coupling to KLH (7). The synthesis was continued on the remaining half by stepwise coupling of amino acids 111 to 120 (H_2N-FERFEIFPKE-COOH) of the hemagglutinin (HA) HA1 polypeptide of influenza virus A/PR/8/34 (H1N1) (1). This sequence represents a determinant able to elicit T-helper cells with a repertoire similar to that of BALB/c mice primed with whole influenza virus (8, 11). Two peptides were thus obtained: a E2-KLH conjugate and a synthetic immunogen containing E2 adjacent to the influenza virus HA T-cell determinant (E2HA).

Four-week-old MHV-seronegative BALB/c mice (13) were injected subcutaneously with 50 μg of S peptides emulsified in complete Freund adjuvant. Three and six weeks later, the animals were boosted with antigen emulsified in incomplete Freund adjuvant. Sera were collected 1 week after each booster immunization and tested for the presence of antibodies by radioimmunoprecipitation analysis

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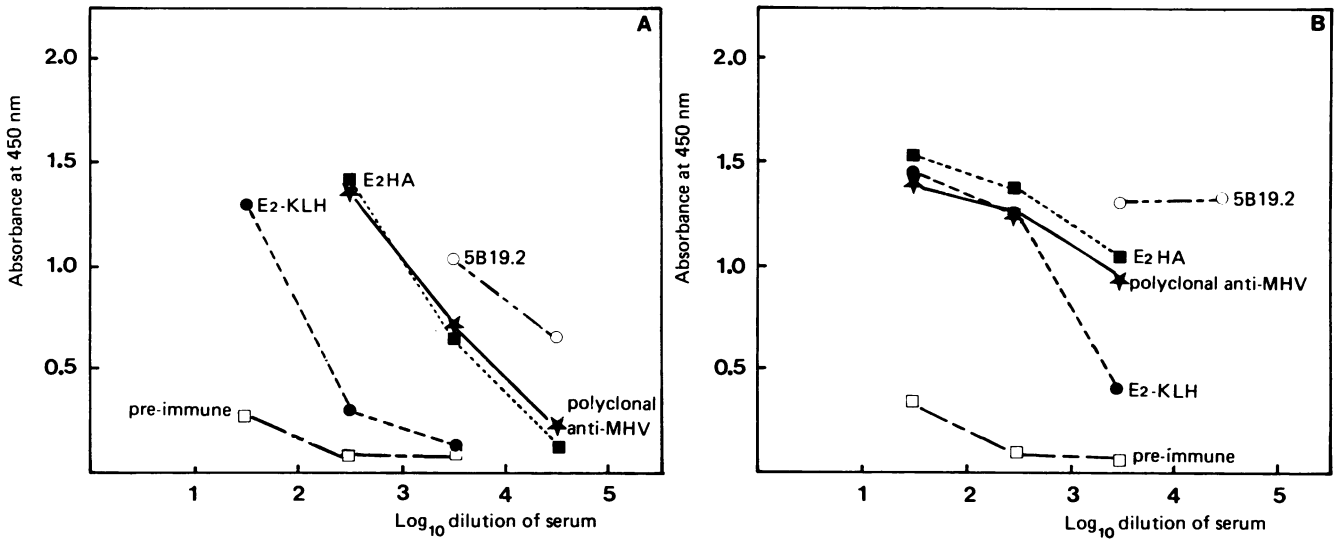

FIG. 1. Binding of antibodies to microtiter plates coated with E2HA peptide (A) or sucrose gradient-purified virus (B). Standard deviations of triplicate samples were less than 5% of the mean.

(14), plaque reduction (13), and enzyme-linked immunosorbent assays.

The copolymeric peptide E2HA and the conjugated peptide E2-KLH resulted in antibody production (Fig. 1). The E2HA peptide induced a 10-fold-higher titer than the corresponding KLH-conjugated peptide (Fig. 1A). As expected, MAb 5B19.2 directed against the S protein of MHV-4 also recognized the E2HA peptide. Sera collected from mice which survived an acute infection with MHV-A59 wild-type virus also recognized the E2HA peptide. The anti-S peptide antibodies also recognized native virions (Fig. 1B). That the HA sequence of the E2HA peptide behaved as a T-cell helper determinant was shown in T-cell proliferation assays in vitro. Splenocytes from mice immunized with the E2HA peptide reacted specifically with E2HA (stimulation index [SI] of 4) and concanavalin A (SI of 8.5) and did not react with the E2 peptide of 13 amino acids (SI of 0.9) or purified MHV-A59 virions (SI of 1.0) in a 72-h proliferation assay. Splenocytes from control mice immunized with adjuvant alone did not respond to the E2HA peptide, but they demonstrated strong reactivity to concanavalin A (SI of 7.4).

As demonstrated by radioimmunoprecipitation analysis (14), the anti-S peptide response had indeed been elicited by the peptide immunization and not by an accidental intercurrent MHV infection (Fig. 2). The intracellular precursor of the S protein (gp150) was precipitated by the anti-E2HA antiserum, which, on the other hand, did not recognize the N and M proteins. The reference lanes showed precipitation of the S and N proteins by the polyclonal hyperimmune serum; the M protein was hardly detectable, probably due to heating of the samples before gel electrophoresis. A cellular 200-kilodalton protein was nonspecifically precipitated and observed in all lanes.

Antigen-antibody immunoglobulin G reactions were measured by competition enzyme-linked immunosorbent assays with serial dilutions of the S peptide in the presence of a fixed concentration of preimmune serum (diluted 1/500), anti-E2HA hyperimmune serum (diluted 1/500), or MAb 5B19.2 (diluted 1/30,000). Binding of both the anti-E2HA antibodies and MAb 5B19.2 to MHV was inhibited by the E2 peptide (13 amino acids) (Fig. 3). At a peptide concentration

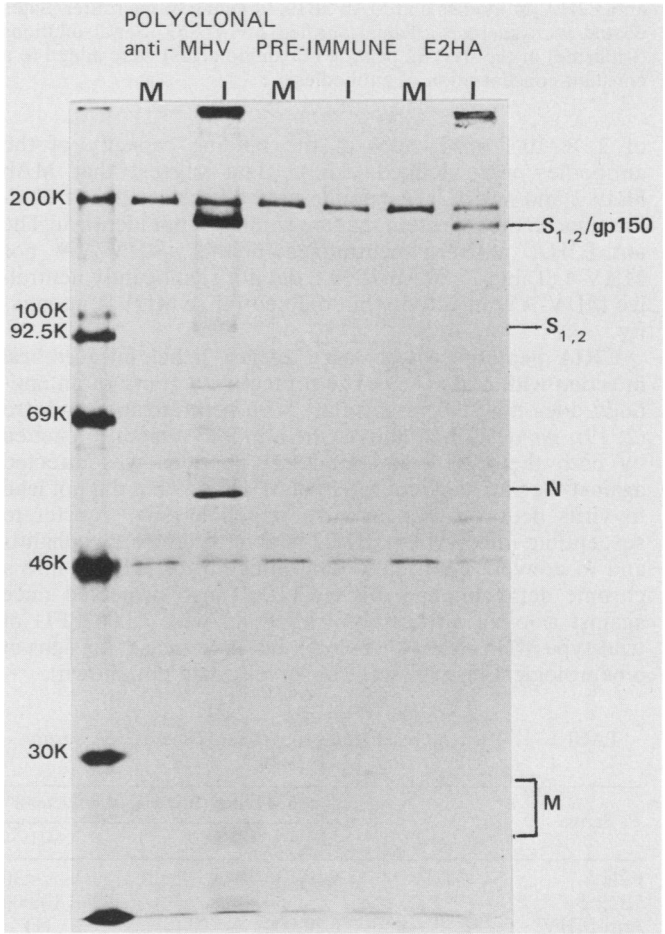

FIG. 2. Specificity of anti-E2HA peptide and polyclonal anti-MHV sera as demonstrated by immunoprecipitation and analysis in a sodium dodecyl sulfate-12.5% polyacrylamide gel. Infected (I) and mock-infected (M) monolayer cultures of Sac(−) cells were labeled with [^{35}S]methionine. Exposure was for 3 days. K, Kilodaltons.

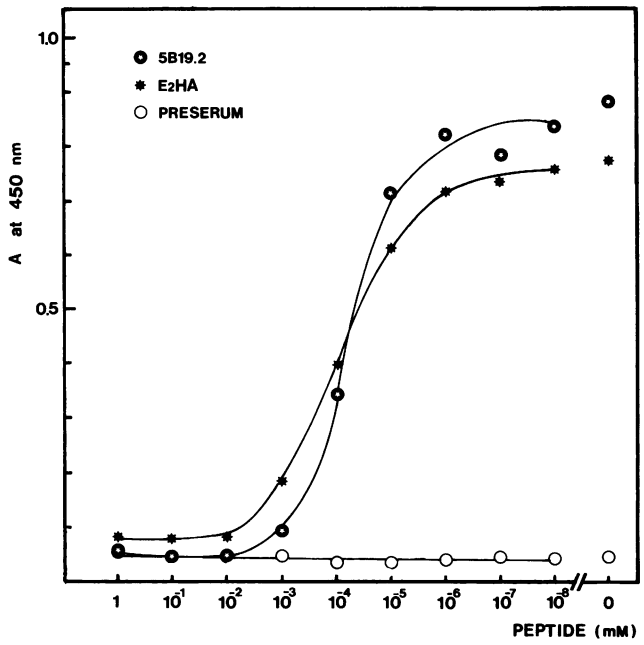

FIG. 3. Competition enzyme-linked immunosorbent assays with anti-E2HA antibodies and MAb 5B19.2 binding to microtiter plates coated with sucrose gradient-purified MHV-A59. Serial dilutions (triplicate) of the free E2 peptide (13 amino acids) were added to a constant concentration of antibodies.

of 2×10^{-5} mM, 50% of the binding capacity of the antibodies was blocked. These data suggest that MAb 5B19.2 and anti-E2HA peptide antibodies recognized binding sites on the S protein that are similar, if not identical. The anti-E2HA antisera neutralized neither MHV-A59 nor MHV-4 (Table 1). MAb 5B19.2 did not significantly neutralize MHV-A59 infectivity but did neutralize MHV-4 infectivity.

E2HA peptide protects mice against lethal intracerebral infection with MHV-A59. The protective activity of an antibody does not always correlate with neutralization in vitro (2, 19). As described above, the antibody response elicited by both the E2HA and E2-KLH peptides was directed against the native glycoprotein of MHV-A59 but did not lead to virus neutralization in vitro. Upon passive transfer to susceptible mice, MAb 5B19.2 is able to block encephalitis and to convert a normally fatal infection of MHV-4 into a chronic demyelinating disease (2). It also protected mice against a lethal intracerebral infection with 1,000 PFU of wild-type MHV-A59 (see below), but in this case, no signs of a neurological disease were observed (data not shown).

TABLE 1. Virus-neutralizing properties of anti-E2HA serum and MAb 5B19.2

Serum	Antibody titer (50% plaque reduction)[a]	
	MHV-A59	MHV-4
E2HA	<30	<30
5B19.2	50	10,000
Anti-MHV[b]	20,000	NT[c]
Preimmune	<30	<30

[a] Detection level was <30. Reciprocal serum dilution giving 50% virus neutralization.
[b] After challenge.
[c] NT, Not tested.

TABLE 2. Result of MHV-A59 wild-type virus intracerebral challenge in BALB/c mice immunized with the synthetic peptides E2HA and E2-KLH

Antigen	Dose (μg/animal)[a]	Mortality/ no. of mice	Antibody titer[b]
E2-KLH	50	5/5	85
E2HA	50	1/15	1,270
Control		10/10	<30

[a] Boosted at 3 and 6 weeks after priming.
[b] Calculated as the reciprocal serum dilution giving an A_{450} of 1.0 in an enzyme-linked immunosorbent assay coated with MHV-A59 wild-type virus.

We then asked whether mice immunized with the S peptides were protected against an intracerebral challenge with a lethal dose of MHV-A59. A dose of 1,000 PFU of wild-type virus (100 to 1,000 50% lethal doses) was injected 2 weeks after the last booster injection. All animals showed clinical signs of an MHV infection; however, 14 of 15 mice immunized with the E2HA peptide were protected, whereas the groups immunized with either the E2-KLH conjugate or with adjuvant succumbed (Table 2). Protection in the absence of neutralizing antibodies was also obtained after immunization with the β-galactosidase-S fusion protein pEX-14, a bacterial expression product that comprises the binding site of MAb 5B19.2 (16; data not shown).

Our data show that immunization with a synthetic peptide with a sequence derived from the S protein induces a protective immune response in BALB/c mice. The mechanism of protection is still unclear. Virus neutralization in vitro certainly is not a faithful reflection of the mechanism by which antibodies elicited with synthetic peptides or MAb 5B19.2 itself prevent an acutely fatal MHV-A59 infection. The S proteins of MHV-A59 and MHV-4 are identical at amino acid positions 798 to 929 (17). In vitro, MAb 5B19.2 neutralizes MHV-4 very well but neutralizes MHV-A59 only poorly. This can be due to either differences in glycosylation of S (at amino acid position 844, a potential glycosylation site) or potential differences in protein folding and the influences of sequence changes in other parts of the S molecule.

Passive transfer studies were performed to examine the quantity of antipeptide antiserum required for protection of mice against a lethal MHV-A59 infection. Groups of 10 mice were intravenously injected with MAb 5B19.2 ascites fluid, anti-E2HA peptide hyperimmune serum, or preimmune serum (100 μl per mouse). MAb 5B19.5 protected all mice, and 30% of the animals receiving a single dose of anti-E2HA peptide antiserum were protected, whereas none of the control group survived when challenged intracerebrally with 1,000 PFU of MHV-A59 wild-type virus. The amount of anti-E2HA antibodies in a 25- to 30-g mouse is approximately equivalent to a 1/300 to 1/400 serum dilution (3 to 4 μl/g). Consequently, the protective immune response following immunization with the E2HA peptide was dose dependent. Similar observations have been made in passive protection studies with monoclonal antibodies to MHV-4 (JHM strain) (2, 5) and MHV-3 (15).

Our studies demonstrated that mice can be protected against a lethal MHV infection by immunization with a synthetic peptide comprising an MHV-A59 B-cell epitope and an influenza virus T-cell epitope. Both antibody-dependent cellular cytotoxicity (10, 15) and natural cytotoxicity against MHV by a subpopulation of B lymphocytes have been advocated as potential mechanisms of protection (9, 26); the latter mechanism of protection is more likely asso-

ciated with recognition of the B-lymphocyte surface by the MHV glycoprotein S, as demonstrated in blocking studies with an anti-S MAb. We therefore anticipate that the protective immune response following immunization with the E2HA peptide is probably mediated through an antibody-dependent cellular cytotoxicity. We are currently investigating the proposed mechanism of antibody-mediated protection in this MHV natural host model.

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